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Overview: Generation of Gene Knockout Mice

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Abstract

The technique of gene targeting allows for the introduction of engineered genetic mutations into a mouse at a determined genomic locus. The process of generating mouse models with targeted mutations was developed through both the discovery of homologous recombination and the isolation of murine embryonic stem cells (ES cells). Homologous recombination is a DNA repair mechanism that is employed in gene targeting to insert a designed mutation into the homologous genetic locus. Targeted homologous recombination can be performed in murine ES cells through electroporation of a targeting construct. These ES cells are totipotent and, when injected into a mouse blastocyst, they can diffentiate into all cell types of a chimeric mouse. A chimeric mouse harboring cells derived from the targeted ES cell clone can then generate a whole mouse containing the desired targeted mutation. The initial step for the generation of a mouse with a targeted mutation is the construction of an efficient targeting vector that will be introduced into the ES cells.

Keywords

gene targeting; homologous recombination; gene knockout

INTRODUCTION

The process of gene targeting provides a means to alter a specified gene in order to better discern its biological role. Through homologous recombination, an engineered mutation can be directed to a designated genetic locus. In this manner, a potentially important genomic clone can directly be utilized to create a mutation into a selected gene. Even amongst the 2.5 Gb of the mouse genome, the cellular DNA repair mechanisms are able to align a targeting vector with its corresponding region of homology and cause recombination into the chromosome. While the goal of transgenic technology is to overexpress a gene to study its biological role in vivo, homologous recombination is typically employed to create a ‘loss of function’ mutation. The most common application of gene targeting is to produce knockout mice, where a drug resistance marker replaces an essential coding region in a genetic locus. In the majority of cases, the importance of a gene cannot be determined by simply recognizing amino acid motifs in the protein (Iredale, 1999). Additionally, the role of a gene often cannot be completely revealed by examining closely related family members. So, gene inactivation is the best way to delineate the biological role of a protein and gene targeting is a direct means to disrupt a gene’s open reading frame and block its expression in a mouse. Not surprisingly, during the twenty years that gene targeting techniques have been available, thousands of genes have been

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knocked out. To date, about 11,000 genes have been knocked out in mice, which accounts for roughly half of the mouse genome (Vogel, 2007; Sikorski and Peters, 1997). Through a combination of gene targeting and gene trapping, a global effort is underway to make a knockout mouse for all of the 25,000 mouse genes (Grimm, 2006).

The knockout mouse has been a valuable tool for geneticists to discern the role of a gene in embryonic development and in normal physiological homeostasis. Mice act as a good analogue for most human biological processes since both species share about 99% of the same genes (Capecchi, 1994). Additionally, mice are useful experimental animals because they are small, have relatively short life spans, and are prolific. So, for geneticists, the targeted deletion of a gene in a mouse provides an important means to determine the biological role of a genetic allele. While useful to study in vivo gene function, some knockout mice have also additionally served as valuable animal models for human genetic diseases. When a human mutation is found to disable a protein, the corresponding knockout mouse can be an important resource to study the underlying pathophysiology and to develop therapies to treat a genetic disease (Majzoub and Muglia, 1996). Additionally, pharmaceutical companies obtain clues about inhibiting a protein by first looking at the phenotype of a knockout mouse (Zambrowicz and Sands, 2003). Thus, knockout mice can provide insight into a gene's physiological role in humans.

Rather than just inactivate a gene, however, some genetic diseases result in the expression of a mutated protein. Point mutations, micro-deletions, or insertions are often responsible for many human genetic diseases. These subtle mutations can also be mimicked in a mouse model using gene targeting. Instead of disrupting a gene, as in most knockout mice, homologous recombination is employed to swap the normal copy of an exon with a mutated version. As long as a similar mutation can be reproduced in the mouse protein, then the corresponding amino acid substitution, deletion, or insertion can be targeted into a gene of interest to replicate the human disease. The effects of the altered protein can then be studied in the animal model.

With some knockout mouse models, the severity of the phenotype can preclude analysis of a gene's role in the organogenesis of a particular tissue. For example, about 15% of all knockout mice have mutations that result in developmental lethality (www.genome.gov/12514551). To circumvent this problem, Cre/loxP technology has been employed to create conditional knockout mice. Derived from the P1 bacteriophage, the Cre recombinase will excise any region of DNA placed between two loxP sites (locus of X-ing over) (Sauer and Henderson, 1998; Sternberg and Hamilton, 1981). The loxP site is a 34 bp nucleotide sequence that can be genetically targeted around an essential exon in a gene (Gu et al., 1994). The resulting mice contain the floxed (flanked by loxP sites) allele in all tissues but are phenotypically wildtype. These floxed mice are then bred to Cre expressing transgenic animals, wherein the promoter used to drive Cre expression will determine the site of the gene deletion. For spatial inactivation of a gene in a mouse, a cell type specific promoter is used to limit Cre expression to a particular tissue. Although less commonly used than Cre/loxP technology, the Flp recombinase also provides a similar means to rearrange a genetic locus. Flp (flippase) was isolated from Saccharomyces cervisiae and, like Cre, the recombinase will also excise DNA flanked between 34 bp sequences known, in this case, as FRT sites (Dymecki, 1996). So, through the use of either the Cre/loxP or the Flp/FRT systems, gene expression can be disrupted in a spatial and temporal manner and the lethality of a knockout mouse phenotype can be overcome. With this versatility, mice utilizing Cre/loxP or the Flp/FRT systems are often shared amongst research laboratories studying differing physiological systems.

In addition to spatial excision of a floxed allele, temporal control of Cre-mediated recombination is also possible in a conditional knockout mouse. The timing of recombination can be regulated by use of a tamoxifen-inducible Cre (Feil et al., 1997; Hayashi and McMahon, 2002). In this strategy, Cre is ligated to a mutated ligand binding domain of the estrogen receptor that restricts transcription until tamoxifen is present. Cre is, therefore, not expressed until tamoxifen is applied either topically or through injection. Another inducible Cre system takes advantage of the reverse tetracycline-controlled transactivator (rtTA) (Utomo et al., 1999). Doxycycline is administered to activate the rtTA that, in turn, will induce transcription of Cre. Lastly, Cre can also be delivered through injection of a viral vector (Anton and Graham, 1995). When and where the Cre is expressed is controlled by the timing and site of injection for the virus. The amount of DNA rearrangement can be adjusted by varying the titer of the virus. The numbers of conditional knockout mice have dramatically increased since Cre/loxP and Flp/FRT technologies were introduced into gene targeting. This proliferation of conditional animal models attests to the value of the recombinases as a molecular switch.

Another interesting application of gene targeting is knock-in technology, in which any gene of interest can be placed under the cis-acting regulatory elements of another gene (Cohen-Tannoudji and Babinet, 1998). Originally, knock-in mice were derived as a means to visualize a gene's expression due to targeted recombination of the lacZ reporter gene into a genetic locus (LeMouellic et al., 1990). The initial coding sequence of a gene would be replaced with the lacZ marker that is inserted under the direction of the gene's promoter. The strategy of using homologous recombination to knock-in a reporter gene, like lacZ, allows for not only the creation of homozygous null mice for a gene, but also provides a technique to study the targeted gene's expression in the heterozygous mice that are often phenotypically normal. The knock-in idea was later elaborated to include the replacement of a gene for the sequence of a similar isoform of the protein (Hanks et al., 1995). Therefore, similar protein isoforms could be tested for redundancy. Through knock-in mice, genetically similar proteins can be examined to determine if two isoforms are truly biologically distinct or if the proteins are basically functionally equivalent with differing patterns of expression being the only key divergence.

VECTOR DESIGN

Homologous recombination takes advantage of a cell's own DNA repair machinery to replace a targeted genetic locus with homologous genomic sequence. The targeted event probably occurs through strand transfer by the recombination RAD52 epistasis group (Vasquez et al., 2001; Rijkers et al., 1998). For recombination to occur at all in a cell, around 2 kb of sequence homology is required (Melton, 2002). However, 6 to 14 kb of homology is typical for targeting constructs. In addition, linear DNA was found to be the preferred substrate for the recombination proteins (Hasty et al., 1992). The actual targeted event takes place in only a small percentage of cells, as homologous recombination occurs about thousand fold lower than random insertions (Sargent and Wilson, 1998). During a stem cell experiment, only about 10^{-2} to 10^{-3} of the DNA integrations are homologous recombination events (Melton, 2002). Therefore, a thorough screening process by Southern blot or by PCR is necessary to identify cells with the targeted event. Electroporation became the preferred way to deliver the replacement vectors into a large number of stem cells. While microinjection has a better targeting ratio (1:15 targeted recombinants to random integrants), a mass delivery system was needed to introduce the targeting constructs into cells and electroporation provided the most efficient technique (1:2,400 targeting ratio) (Vasquez et al., 2001). However, since the transformation efficiency is low (10^{-3}), a positive selection marker is needed to enrich clones that have inserted the targeting vector into their genome (Ledermann, 2000). The likelihood of recombination peaks when cells are in early to mid-S phase and the process occurs rapidly, in only about 30 minutes after the construct is taken into the nucleus (Wong and Capecchi, 1987; Cappecchi, 1989). The rate of homologous recombination is not dependent on the input concentration of vector used in the targeting experiment (Thomas et al., 1986; Capecchi, 2005). So, the capacity to locate homologous sequence within the recipient cell genome is not a rate-limiting step since this would be influenced by the input concentration of DNA. Rather, the cellular machinery performing the homologous recombination sets the reaction rate for gene targeting.

Primarily, two classes of vectors have been used to generate targeted mutations. Most gene targeting experiments employ replacement vectors (Fig. 1), which have been particularly instrumental for efficiently generating knockout mice. In the design of a replacement vector, the open reading frame of a genomic clone is disrupted by the placement of an intervening drug selection marker. Two homologous recombination events function to insert the targeting construct containing the drug resistance gene into a homologous genetic locus (Fig. 2). The drug resistance gene works for the positive selection of cells that have integrated the targeting vector into their chromosome. Neomycin is the most common drug used for positive selection. Integration of the neomycin phosphotransferase (neo^r) gene allows for resistance to neomycin, an aminoglycoside that interferes with protein synthesis in eukaryotic cells. Other drug selection markers include resistance genes for puromycin and hygromycin. Mansour et al. (1988) established positive and negative selection for gene targeting by the additional placement of the HSV thymidine kinase (HSV-tk) gene adjacent to one of the vectors arms containing targeted homology to a genetic locus. Random integrants will usually contain an intact of the HSV-tk gene when inserted into the genome. Cells with random integrants are killed during negative selection through treatment with gancylovir or FIAU (1-[2′-deoxy-2′-fluoro-β-D-arabinofuranosyl]-5-iodouracil), compounds that require phosphorylation by HSV-tk to inhibit DNA synthesis. About a 2,000 fold enrichment of targeted clones can occur with this type of vector. Even with positive and negative selection, a substantial number of ES cell clones that arise in culture are false positive since HSV-tk can be inactivated before recombination by events like partial deletions. The random integrants are, therefore, insensitive to negative drug selection. To avoid subjecting the ES cells to drugs like gancyclovir and FIAU, some targeting vectors omit using HSV-tk in favor of a a negative selection marker like the diphtheria toxin gene (Yagi et al., 1990). In a gene targeting experiment, a replacement vector directs the disruption of an essential coding exon/s of a gene through the insertion of a positive drug resistance marker. This replacement occurs via recombination at the two flanking homology arms. Any important coding region that is essential for a gene’s function can be thereby targeted for deletion.

When designing a targeting construct, a few factors should be considered that could result in an incomplete knockout. A gene may be residually expressed if there exist alternative or cryptic promoters that are not disrupted in the targeted allele (Müller, 1999). Differential splicing could also generate RNA species where the selection marker is skipped. Read-through transcription of the drug resistance gene is another way for the appearance of mutant mRNA that has some coding sequence from the targeted allele. In gene targeting via a replacement vector, a strong promoter is typically used to drive the neo^r marker in the stem cells, but the polyA addition site can sometimes be skipped during transcription of the drug resistance gene. Therefore, as the neo^r gene is transcribed, downstream exons could possibly be spliced into the mRNA. The neo^r gene is often oriented in the opposite direction of the gene transcription for the targeted allele to avoid any potential transcription of downstream exons. In addition, this orientation also ensures that the strong promoter on the neo^r gene does not influence any downstream genes, as well. Therefore, the size and location of the targeted deletion should be carefully considered when designing a replacement vector to help avoid an incomplete knockout of the gene function.

Double replacement vectors are a variation of the knockout vector design that is primarily used to target subtle mutations into a selected genetic allele (Askew et al., 1993; Stacey et al., 1994). Also known as ‘tag and exchange’, this strategy requires two rounds of homologous recombination to create the desired mutation. In contrast to a replacement vector, the HSV-tk gene is placed adjacent to neo^r rather than at the end of the construct. Both the positive and negative selection markers are inserted into the targeted genomic site with the homology arms flanking both genes. In the first ‘tag’ step, a targeted stem cell clone is isolated with only neomycin treatment. Once isolated, the ES cells then undergo a second round of electroporation with a targeting vector that will ‘exchange’ both drug selection markers for a mutated exon. This second targeting vector is basically a genomic clone containing the desired point mutation, deletion, or insertion. During this second round of gene targeting, gancyclovir is added to isolate cells that have lost the HSV-tk gene from homologous recombination with the second vector. The hprt gene (hypoxanthine-guanosine phosphoribosyl transferase) can be used to replace both the neo^r and HSV-tk genes in the construct design. The hprt gene is a part of a purine salvage pathway that can be adapted as a drug marker to both positive and negative selection (Melton, 2002). HAT (hypoxanthine, aminopterin, thymidine) medium is used for positive selection since it forces a cell to depend on the salvage pathway that needs an intact copy of the hprt gene. Negative selection involves the purine analog 6-thioguanine that is phosphorylated by the hprt gene to generate a toxic compound for the cell. ES cells with a mutated copy of their endogenous hprt gene, however, are needed for this type of strategy. So, with a double replacement vector, two rounds of targeted recombinations are required to obtain the final ES cell clones with the expected subtle mutation. Several mutations can readily be placed into the modified gene once targeted ES cell clones from the first electroporation are isolated.

Subtle mutations can also be introduced with vectors that take advantage of Cre/loxP technology (Fig. 3) (Cohen-Tannoudji and Babinet, 1998;Ferradini et al., 1996). Similar to a replacement vector, the targeting construct is designed with a drug selection marker flanked by two homology arms. Both positive and negative selection is required to isolate cells with the properly recombined mutation. Instead of replacing an entire exon with a drug selection marker, the goal, here, is to exchange normal coding sequence in a targeted allele for a mutated version. One homology arm will carry the planned point mutation, micro-deletion, or insertion to be introduced into the targeted gene. However, in this strategy, the positive drug selection marker needs to be removed because it will interfere with transcription of the mutated allele. Using a neo^r gene that is floxed allows a way to eventually remove the drug selection marker by using Cre recombinase. In the construct design, the neo^r gene is inserted into a non-coding region. Upon recombination, only a 34 bp loxP site remains in the intron to create a ‘clean’ mutation (without a drug selection marker) in the targeted genetic locus. Without disruption by a drug selection marker, the modified allele should be transcribed to produce the expected mutant protein, and the remaining loxP site within an intron should not interfere with gene expression. Like double replacement vectors, this strategy is also a two-step process, except that Cre is used to remove the drug selection marker rather than use a second round of homologous recombination.

Making a targeting construct for a conditional knockout is essentially like making a ‘clean’ mutation, but, with this strategy, the inserted mutation results in the addition of loxP sites into a targeted genetic locus (Fig. 4). In the final targeted allele, two loxP sites will flank an essential exon/s that is to be excised by Cre in a conditional manner (Gu et al., 1994;Cohen-Tannoudji and Babinet, 1998). As with the ‘clean’ mutation, a floxed neo^r gene is needed for positive selection and is eventually be removed using Cre. This process of Cre excision will leave one of the necessary loxP sites required to flox a targeted exon/s. One of the homology arms from the replacement vector is then used to insert the other flanking loxP on the opposite side of this exon/s. Both loxP sites must be positioned in the introns of the targeted gene and should not interfere with the either the coding sequences or the promoter of the gene nor with any neighboring genes. After homologous recombination, the mutated genetic locus will initially have three loxP sites consisting of the floxed exon/s followed by a floxed neo^r gene. Once the targeted clones are identified, the targeted ES cells are then transiently transfected with a Cre expression vector to remove the neo^r gene. Three scenarios arise when Cre is used to recombine the targeted allele. In one situation, the targeted allele is properly recombined and Cre only excises the neo^r gene. However, recombination can also result in the exclusion of the floxed exon/s or both the exon/s and the neo^r gene. A second round of genomic screening by Southern blot or PCR is needed to find the ES cell clone that is missing the neo^r gene but has the floxed exon intact. Transcription of the targeted gene should not be impacted when the final floxed allele is generated. The mutated mice are then bred to a Cre expressing transgenic line to allow for conditional disruption of the gene in the tissue of interest.

Knock-in strategies basically adapt replacement vectors to usurp the endogenous promoter of a gene to transcribe an exogenous cDNA of interest (Fig. 5) (Cohen-Tannoudji and Babinet, 1998;LeMouellic et al., 1990;Hanks et al., 1995). During recombination, the protein start site of the targeted gene is disrupted when a selected cDNA is knocked into the promoter, basically resulting in a targeted transgenic mouse. For this vector design, both the cDNA to be knocked-in and the neo^r gene are brought into the genome by two flanking homology arms. The 5’ arm, in this case, will typically contain homologous genomic sequence that is upstream from the protein start site. Similar to a transgenic construct, this regulatory sequence is followed by a cDNA and a poly A addition signal. For positive selection, a drug resistance gene like neo^r is placed downstream of the cDNA. A second 3’ homology arm then follows after the neo^r gene. Like a replacement vector, two homologous recombination events function to replace the coding sequence of the gene with the cDNA and neo^r drug marker in the construct. If positioned correctly, the knocked in cDNA should then be in frame and expressed by the recombined gene. Through this design, a gene can be knocked out as the cDNA of closely related isoform is simultaneously knocked-in. Protein domains can be switched by homologous recombination as well. In addition, marker genes, like lacZ, can be knocked in not only to produce a null mouse, but also to help track the targeted gene’s expression pattern through the mutated gene. Even though knock-in mice are more time consuming to generate than transgenic mice, the knocking-in of a cDNA into a targeted genetic locus can be useful to overcome problems of unreliable transgene expression that sometimes occur from the randomness of the site of integration and the variability in transgene copy number (Ledermann, 2000;Jasin et al., 1996). If the knocked-in cDNA is correctly in frame, expression of the knocked in transgene should more faithfully mirror the transcription pattern for the targeted promoter. In one such example, Cre was targeted to the Cd19 genetic locus in order to maintain all of the regulatory elements for transcription in B cells (Rickert et al., 1997).

Lastly, gene targeting with a replacement vector can be applied to generate a single copy transgenic mouse. The use of hprt null ES cells allows for only a single copy of a transgene to be integrated at a known insertion site (Bronson et al., 1996). With this replacement vector design, homologous recombination is needed to correct the hprt gene. The homology arms of the targeting construct restore two missing exons needed for a functional copy of the hprt gene during recombination in the stem cells. During homologous recombination, a single copy of a transgene is carried into the restored hprt genetic locus. The transgene flanked by the targeting arms has its own promoter, selected cDNA, and poly A addition site. With reconstitution of the hprt gene available as a drug selection marker, clones with the proper homologous recombination can then be isolated with HAT medium.

Another less commonly used gene targeting strategy employs insertion vectors to disrupt a genetic locus (Fig. 6). These insertion vectors are designed using just one arm of homologous sequence and a single recombination event is all that is required to insert a drug selection gene like neo^r into the targeted gene (Hasty et al., 1991). A restriction enzyme site located within the homology arm is used to linearize the construct so that homologous recombination to occur. Instead of replacing sequence, the whole insertion vector is integrated into the genome. In this case, the homology arm serves to merely direct the site of integration to the targeted gene. Insertion vectors result in gene duplication during homologous recombination because the entire targeting construct is inserted where the homology arm was linearized. In most cases, the neo^r gene will interfere with transcription in the targeted gene to make a 'loss of function' mutation. However, since homologous recombination with an insertion vector produces gene duplication, mutant RNA species may still be expressed through altered splicing. In addition, intrachromosomal recombination can also lead to regeneration of the wild type allele. Compared with replacement vectors, though, constructs of this type have a higher frequency of recombination (Hasty et al., 1991). A variation of the insertion vector strategy is to create a subtle mutation through a 'hit and run' or 'in-out' strategy (Vanlancius and Smithies, 1991). With this technique, the homology arm contains a desired mutation to be inserted into the targeted gene. Through homologous recombination, both the neo^r and HSV-tk genes are inserted into the targeted gene. After initial positive selection with neo^r, the isolated cells then undergo a second round of drug selection with gancyclovir. During this next step, the duplicated gene should align in such a way as to undergo intrachromosomal recombination. Both drug selection markers are then be lost in the process. The remaining recombined allele will retain the subtle mutation and the mutated protein will be expressed.

STRATEGIC PLANNING FOR DESIGNING A KNOCKOUT TARGETING CONSTRUCT

While creating a knockout mouse is costly and labor intensive (>$12,000 and ~1 year from electroporation of the targeting vector in ES cells to the generation of homozygous null mice) (www.med.umich.edu/tamc), the understanding of gene functions in mammalian physiology and development have made the efforts worthwhile. To design a vector for gene targeting, the following steps are suggested:

1. After selecting a gene to be disrupted, the genomic structure of the target allele should be researched. Exon/intron sequence information should be gathered. The size of the gene and the chromosomal location of the allele to be targeted should also be known. Lastly, the location of most major restriction enzyme sites should be identified to aid with subcloning. A good restriction enzyme map of the genomic locus will be useful in the construction of the replacement vector. Once an allele is selected for targeted deletion, the flanking genomic sequence should be examined to ensure that any possible neighboring genes are not disrupted during recombination (Olson et al., 1996). Check for potential open reading frames that could be disturbed when a targeted mutation is generated. Most genomic sequence information can be gathered through the Mouse Genome Sequencing Consortium (MGSC). The mouse genome sequence is freely available in public databases (GenBank accession number CAAA01000000) and is accessible through various genome browsers (http://www.ensembl.org/Mus_musculus/; http://genome.ucsc.edu/ and http://www.ncbi.nlm.nih.gov/genome/guide/mouse/) (Mouse Genome Sequencing Consortium, 2002). Lastly, perform a thorough search to ensure that your gene of interest has not been knocked out already. About 700 knockouts have been made three or more times (Grimm, 2006).

2. A mouse genomic fragment containing a large portion of the gene to be targeted needs to be isolated to create the homology arms in the targeting construct. A 129/Sv genomic clone is most commonly used for constructing targeting vectors since most stem cells were derived from this mouse strain. Using a genomic clone of a mouse strain different from the ES cell strain can reduce the frequency of homologous recombination. In general, homologous recombination is four to five times more efficient with targeting vectors using isogenic DNA (Deng and Capecchi, 1992). For any given genetic locus, however, the rate of recombination will depend on the heterology in the mouse strains, as a 20 fold decreased efficiency has been reported

with the use of non-isogenic DNA in the targeting vector (te Riele et al., 1992). Searching a BAC (Bacterial Artificial Chromosome) library is one means to get the genomic sequence needed to make a targeting vector. Due to sequencing of the mouse genome, BLAST can be used to readily find a BAC clone that contains the selected gene for targeted disruption. Genomic DNA from a female 129S6/SvEvTac (Taconic) mouse, for example, was used to create the RPCI-23 BAC library. A BAC clone will generally contain from 100 to 300 kb of genomic sequence. Another means to obtain the homology arms for the targeting vector is through PCR. PCR can be used to amplify the homology arms, if a source of purified 129/Sv genomic DNA is available. A high-fidelity Taq polymerase is needed that can amplify long stretches of genomic DNA. Even with a high-fidelity Taq polymerase, however, the PCR products must be carefully sequenced to ensure no coding errors were generated during the reaction. Lastly, a mouse 129/Sv Lambda phage genomic library is another option to isolate the sequence for the gene to be targeted. A probe containing some homology to the chosen allele is used to screen the phage library. Once a genomic clone is isolated, most of the common restriction enzyme sites should be verified.

The total amount of homology needed in most successful gene targeting experiments is about 5 to 10 kb in length. The efficiency of homologous recombination increases when increasing the length of homology in the targeting construct, but this peaks after 14 kb of sequence (Deng and Capecchi, 1992). For a typical replacement vector, the total length of homologous sequence will consists of roughly a 1-2 kb span of DNA for a short homology arm and a separate larger 4-6 kb genomic fragment as a long homology arm. The short arm can be as small as 0.5 kb, however, without affecting targeting efficiency (Hasty et al., 1991). In the case of the long arm, though, increasing the length of homology from 8 up to 110 kb does not enhance the frequency of homologous recombination (Lu et al., 2003). In the construct, the short and long arms will share homology with genomic sequences that flank the designated coding region to be deleted from the gene to be targeted.

3. Both positive and negative selection genes need be chosen to construct the targeting vector. The most common positive selection marker is the neomycin phosphotransferase (neo^r) gene. The drug resistance genes for puromycin and hygromycin have also been used as positive selection markers. When designing a targeting vector, the expression of the neo^r gene is often inserted in the opposite orientation to transcription in the target allele. With negative selection, the HSV thymidine kinase (HSV-tk) gene is often used to select against random integrants. If the targeting construct is randomly integrated, the cells will mostly contain an intact copy of the HSV-tk gene that will phosphorylate gancyclovir into a cytoxic drug (Mansour et al., 1988). The diphtheria toxin gene has also been used for negative selection against random integrants (Yagi et al., 1990). A frequently used plasmid for making replacement vectors is pPNT (Tybulewicz et al., 1991). This plasmid contains both the neo^r and HSV-tk genes. All that is required is to ligate the two homology arms into the unique restriction enzyme sites that flank the neo^r gene.

With the acquisition of the drug markers and a genomic clone, one can proceed with piecing a targeting construct together.

PROTOCOL FOR DESIGNING A KNOCKOUT TARGETING CONSTRUCT (REPLACEMENT VECTOR)

Equipment

Microcentrifuge

Gel Electrophoresis Equipment

UV Camera Setup

Materials

Genomic Clone (isogenic with ES cell line to be used)

Expression Vector with Positive and Negative Drug Selection (i.e. pPNT [Tybulewicz et al., 1991])

Ligation Kit

Competent Cells

Restriction Enzymes and DNA Modifying Enzymes

Procedure

1. Once a gene is chosen for targeted inactivation, determine the best region of genomic sequence to disrupt through homologous recombination. With a replacement vector, two homologous recombination events will occur at the targeted locus via the long and short homology arms. The positive drug resistance gene is inserted into a gene while coding sequence is simultaneously deleted. The length of genomic sequence replaced by the drug selection marker is governed by the location of these homology arms. So, any intervening sequence situated between the two directed sites of recombination is omitted during the process of homologous recombination. The length of sequence that can be replaced by the positive selection marker does not seem to influence the efficiency of homologous recombination. Up to 15 kb of sequence, for example, was deleted with a replacement vector to generate the T-cell receptor knockout mouse (Mombaerts et al., 1991). With most targeting constructs, however, basically one to a few critical exons are generally disrupted in gene targeting experiments. Small deletions of 0.5 to 1 kb in length are typical for most gene targeting experiments and are usually sufficient to inactivate a gene (DeChiara, 2001). Genetic coding sequence essential for protein function should be selected for targeted deletion because there is the possibility that the inserted drug marker can be bypassed during transcription of the targeted allele. Any resulting truncated protein generated should, therefore, not be functional and would hopefully be rapidly degraded so as not to interfere with the cell's normal physiology. Generally, deleting 5'exons provides a better chance to totally disrupt the formation of protein instead of using a strategy that targets downstream exons at the C-terminus (Hasty et al., 1993). With genes smaller than 20 kb in length, some investigators prefer to design replacement vector that disrupt all the coding exons in order to absolutely ensure gene inactivation (Cheah and Behringer, 2001). For any gene targeting strategy, though, be sure that no neighboring genes are affected during homologous recombination.

2. After designating a location for targeted deletion, identify neighboring restriction enzyme sites that are convenient for generating a short homology arm (~1 to 2 kb in length) as well as the long homology arm (~4 to 6kb). A total amount of 5 to 8 kb of homology is typical in most targeting vectors (Hasty et al., 1993). Both the short and long homology arms may need to be subcloned into a vector such a pBluescript (Stratagene) in order to facilitate in the assembly of the final targeting construct. Often, DNA modifying enzymes are needed with subcloning since convenient restriction enzyme sites may not be readily available in the genomic clone. The following DNA modifications may need to be applied for subcloning the homology arms:

a. Incompatible overhangs produced from a restriction enzyme digest can be blunted to help facilitate the subcloning of a homology arm. Blunt ligatable ends can be generated with enzymes like Mung Bean Nuclease (to remove a 3’ overhang) or Klenow Polymerase (to fill in a 3’ recessed end). However, sticky end modification can result in reduced ligation efficiency.

b. Adaptors can be purchased (New England Biolabs, Stratagene, etc.) to insert a desired restriction enzyme site onto blunt ended DNA. Alternatively, oligonucleotide adaptors can be generated to insert a restriction enzyme site into cleaved DNA with sticky ends. Oligo’s must be designed so that, upon annealing, sticky ends are generated which are compatible to the cleaved DNA. The synthesized oligos are diluted to a concentration of 100 ng/μl in a buffer of 10mM Tris-HCl and 50mM NaCl. This solution is then heated at 95C for 15 minutes and then cooled to room temperature. The annealed oligo’s are ligated with the cleaved DNA at a 500 to 1000 molar excess. This high concentration helps to facilitate ligation of the oligo and inhibits mere religation of the vector.

c. For ligation, the vector may need to be modified to prevent religation, particularly if only one restriction enzyme is being used to cleave the DNA. In this case, the vector needs to be dephosphorylated to prevent religation. Dephosphorylation of the DNA can be achieved by incubating the cleaved vector with an alkaline phosphatase (typically either calf intestinal or shrimp AP).

As mentioned above, PCR provides another means to clone out the homology arms, but careful attention should be made to ensure that no coding errors were generated during the reaction.

3. The targeting construct can be assembled after both homology arms and the drug selection markers have been gathered. The targeting construct should be ligated so that the long and short homology arms flank the positive selection marker. A negative selection marker is subcloned outside of this grouping. Try to ligate the vector so that, upon recombination, the positive selection marker is transcribed in the opposite orientation of the targeted gene. A unique restriction enzyme site must be maintained to linearize the targeting construct to improve the efficiency of homologous recombination (Hasty et al., 1992). This restriction enzyme site should be located outside the regions of homology, typically between the plasmid backbone and a targeting arm. With linearization of the targeting construct, the plasmid DNA will protect the negative selection marker like HSV-tk gene from nucleases (Cheah and Behringer, 2001). The placement of either the long or the short arm between the two drug markers has varied amongst targeting vectors. While some investigators prefer to locate the HSV-tk adjacent to the long targeting arm (Torres and Kuhn, 1997), other vectors place the HSV-tk gene at the end of the short targeting arms because this design may help improve the efficiency of negative selection (Melton, 2002).

Plasmids such as pPNT (Tybulewicz et al., 1991) or the pKO Scrambler Series ρ (Lexicon Genetics) can be useful in designing targeting constructs since these vectors contain both neo^r and thymidine kinase genes. In addition, common restriction enzyme sites are positioned in locations to facilitate the subcloning of the homology arms. With pPNT, for example, one homology arm can be subcloned into the restriction enzyme sites (XbaI, BamHI, KpnI, and EcoRI) located between neo^r and HSV-tk genes. Both of these drug selection marker genes in this vector are driven by the 3-phosphoglycerate kinase promoter (PGK) (McBurnery et al., 1991) with the PGK pA addition site. PGK is a housekeeping enzyme and the promoter is useful to drive high expression of these drug markers. The second homology arm can then be placed adjacent to the neo^r gene with NotI and XhoI restriction enzyme sites. Since NotI is a rare 8 base pair cutter, this site is useful for linearizing targeting constructs. An example of a targeting construct is illustrated in Figure 1.

4. While the replacement vector is being constructed, a strategy to identify the targeted clones can be planned using all the collected sequence information about the genomic clone. Typically, to be certain of proper recombination, a Southern blot test should be designed to identify the targeted stem cell clone. With a Southern blot, an appreciable fragment size difference must appear between the wild type and the mutated allele. An ideal restriction enzyme should be identified that will best show a band difference from the digested genomic DNA. Southern blot analysis usually tests either the insertion or deletion of a restriction enzyme site due to homologous recombination. A fragment of the genomic clone is generally retained to make a Southern probe that contains sequence flanking the homology arms. This ensures that the Southern probe will not bind with random integrants. Therefore, the only bands to be detected with the Southern probe will either be the recombined allele or from the wild type gene. PCR can also be used to find properly targeted clones. PCR can provide a fast high throughput means to test for targeted clones. With PCR, one primer will typically be designed within the neo^r gene and the other primer will be located outside the short arm of the replacement vector. Screening by PCR, however, can be is tricky since long PCR products need to be amplified. In addition, there is always the risk of either false positives or negatives when using PCR to find a clone. Once a positive clone is identified, though, PCR becomes the preferred strategy to genotype the resulting mutant mice.

DESIGNING A KNOCK IN TARGETING CONSTRUCT

Designing a knock-in construct follows the same basic rules as a knockout replacement vector. One homology arm, however, must consist of genomic sequence upstream of the protein initiation site. In this case, the 5’ and 3’homology arms will flank both the knocked in cDNA and a positive drug selection marker. Be sure to include a pA addition signal for the knocked in cDNA. Upon recombination, the promoter in the targeted gene will drive expression of the inserted cDNA (Fig. 5). For efficient homologous recombination, the length of non-homologous DNA (i.e., the cDNA and neo^r gene) inserted into a target locus should not exceed the length of total homology (the long and short arms of the targeting construct).

PROTOCOL FOR DESIGNING A CONDITIONAL KNOCKOUT TARGETING CONSTRUCT

With a conventional knockout vector, an essential coding region in the targeted gene is replaced with a drug selection marker during homologous recombination. For a conditional knockout mouse, however, the final targeted allele needs to be functionally intact. In this case, the end result of gene targeting is the placement of loxP sites around an essential coding region to create a floxed allele. Therefore, no sequence should be omitted by gene targeting. Basically, a continuous length of homologous genomic sequence is ideal in making a vector for a conditional knockout. A fragment of the genomic clone is merely cut in two with a restriction enzyme to derive a long and short homology arm. This design contrasts with a conventional knockout where two separate lengths of homologous genomic sequence are needed to make the targeting vector. One of the homology arms is then modified to position a loxP site next to an essential exon for conditional deletion.

While a positive drug selection marker (i.e., neo^r gene) is needed for initial enrichment of targeted clones, it must be floxed so that it will not interfere with the final mutated gene. Once the targeted clones are identified, this drug marker is removed by transient transfection to express Cre recombinase. If the correct recombination event occurs, then only a loxP site will remain after Cre excises the neo^r gene. In combination with the loxP site in the targeting arm, the exon/s designated for conditional deletion should basically be floxed upon Cre mediated rearrangement. However, Cre recombination can also result in the exclusion of the floxed exon/s or both the exon/s and the neo^r gene, so stem cell clones must be screened to determine the correct rearrangement (Fig. 4).

1. A homologous 129/Sv genomic fragment continuous with the gene of interest needs to be isolated and characterized. The length of genomic DNA needed for a targeting vector follows the same rules listed as a conventional knockout vector, with roughly about 5 to 10 kb of homologous sequence required for efficient recombination. Exon/intron boundaries and restriction enzyme sites need to be mapped. Afterwards, the coding region to be floxed should be determined. The final loxP sites should be located within intronic sequence so that gene expression is not affected. A loxP site should not be designed into any promoter regulatory sequence, as well, for this may interfere with transcription. In the floxed allele, all the coding sequence should be in frame. Again, check for any neighboring open reading frames that could be disrupted after Cre recombination.

2. A plasmid with a floxed neo^r gene should be acquired to create the targeting construct. A negative selection marker should also be obtained such as the HSV-tk gene.

3. Restriction enzyme sites in the genomic clone should be identified to aid in the placement of the loxP sites. It is best if two unique restriction enzyme sites can be found that flank a vital coding region for placement for the floxed neo^r gene as well as a loxP site. As mentioned, the loxP sites should only be placed in intronic sequences. If available, the floxed neo^r gene can be subcloned into one of the unique restriction enzyme sites. In the second unique restriction enzyme site, a synthetic loxP site can then be ligated into the vector. Oligonucleotides with the loxP site and restriction enzyme site overhangs can be synthesized, annealed, and ligated into the vector. The annealed synthetic oligo should be as follows:

   5′—RE Site—ATAACTTCGTATA<u>GCATACAT</u>TATACGAAGTTAT—RE Site—3′

   3′—RE Site—TATTGAAGCATAT<u>CGTATGTA</u>ATATGCTTCAATA—RE Site—5′

   A loxP site consists of two 13 bp inverted repeats that flanks an asymmetric 8 bp core (underlined). The asymmetric core determines the directionality of the loxP site. All loxP sites in the targeting construct must be in the same orientation. Otherwise, if the loxP sites are in opposite orientation, the floxed sequence will be inverted rather than deleted from the gene. To anneal the oligos, dissolve the DNA to form a 100 ng/µl solution in 10 mM Tris-HCl and 50 mM NaCl. Combine sense and anti-sense strands in a microfuge tube, heat at 95 ^{0}C for 15 minutes, then let slowly cool to room temperature for proper annealing. If needed, the vector pBS246 can be obtained to procure the loxP sites. This plasmid has two loxP sites that flank a multiple cloning site (Sauer, 1993). Sequence can then be ligated around the loxP sites to get the desired floxed allele.

4. After proper placement of the floxed neo^r gene and a loxP site within the homologous genomic fragment, this cluster should then be ligated together with a negative selection marker like HSV-tk. Again, a unique restriction enzyme site must be located adjacent to the homology arm so that the targeting vector can be linearized.

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5. Eventually, after electroporation and isolation of targeted clones, the floxed neo^r gene should be removed by transient expression of Cre. Therefore, a Cre expression vector must be acquired for generation of the final mutated allele. One such plasmid is pBS185 (Sauer and Henderson, 1990). This vector will express Cre under the CMV promoter.

6. A PCR protocol should be designed to identify the floxed allele. With the sequence information from the genomic clone, primers can be designed to detect wild type, floxed, and Cre recombined alleles. If possible, cell culture work should be performed to test Cre excision and PCR analysis before the actual electroporation of the targeting construct into the stem cells.

History

Two discoveries have been instrumental for the ability to generate knockout mice, the isolation of stem cells and the discovery of homologous recombination. The significance of these findings was demonstrated when Mario Capecchi, Oliver Smithies, and Martin Evans were awarded the 2007 Nobel Prize in Physiology or Medicine for their work in establishing the knockout mouse model (Vogel, 2007). The key step needed for making knockout mice was the isolation of embryonic stem cells (ES cells). The ES cells were isolated from the inner cell mass of a 3.5 days postcoitum mouse blastocyst (Evans and Kaufman, 1981; Martin, 1981). These stem cells could progressively grow in tissue culture and were pluripotent. By injecting the stem cells into a mouse blastocyst, Bradley et al. (1984) were able to generate chimeric mice. The production of chimeric mice proved that the stem cells were able to differentiate into multiple cell lineages and were able to contribute to the development of the mouse embryo. Germline transmission was also achieved by using the pluripotent ES cells. After breeding the chimeric mice, the resulting offspring that were born were clearly derived from the ES cells as seen with the transmission of coat color. Therefore, the introduced stem cells could become established into the germline of the chimeric mice. The isolation of stem cells basically meant that an ES cell clone that was genetically modified in culture could eventually be used to derive a mouse.

The second important step in the establishment of knockout mice was the discovery of homologous recombination within mammalian cells. Lin et al. (1984) were first able to demonstrate that homologous recombination was feasible by reconstructing a functional thymidine kinase gene in mouse L cells. Later, Smithies et al. (1985) were able to modify the β-globin locus with the insertion of the neomycin resistance gene. This work provided a means to specifically target the DNA insert to a planned genetic locus, in contrast to the random integration of a construct that is used with transgenic technology.

The convergence of these two technologies provided the means to generate knockout mice. The first ideal genetic locus to test gene targeting was for the enzyme hypoxanthine-guanosine phosphoribosyl transferase (hprt). Loss of the hprt could be tested with treatment using 6-thioguanine, while restoration of the gene can be selected in hprt null cells with addition of hypoxanthine, aminopterin, thymidine (HAT) medium. In addition, the hprt gene is X-linked, so, with male stem cells, only one allele needed to be targeted for drug selection. Doetschman et al. (1987) proved that a mutant hprt gene could be corrected through homologous recombination in ES cells while Thomas and Capecchi (1987) demonstrated targeted gene disruption. These experiments lead the way to target non-selectable genes starting with int-2 and c-abl knockout mice (Mansour et al., 1988; Schwartzberg et al., 1989). Since the first knockouts, there has been an explosive growth in the numbers of animal models derived through the technique of gene targeting. While mouse stocks with spontaneous or radiation-induced mutations existed in the past, the mutated alleles did not correspond to the majority of biologically important cloned genes (Mak, 1998). Through homologous recombination, a cloned gene could be directly utilized to disrupt the target allele. Currently, a targeting strategy is often planned shortly after the mouse gene is originally cloned. Therefore, targeted recombination has become well established as an important tool to inactivate a gene in order to study its function in vivo.

References

Anton M, Graham FL. Site-specific recombination mediated by an adenovirus vector expressing the Cre recombinase protein: a molecular switch for control of gene expression. J. Virol 1995;69:4600–4606. [PubMed: 7609024]

Askew GR, Doetschman T, Lingrel JB. Site-directed point mutations in embryonic stem cells: a gene-targeting tag-and-exchange strategy. Mol. Cell. Biol 1993;13:41115–4124.

Bradley A, Evans M, Kaufman MH, Robertson E. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature 1984;309:255–256. [PubMed: 6717601]

Bronson SK, Plaehn EG, Kluckman KD, Hagaman JR, Maeda N, Smithies O. Single-copy transgenic mice with chosen-site integration. Proc. Natl. Acad. Sci 1996;93:9067–9072. [PubMed: 8799155]

Capecchi MR. The new mouse genetics: altering the genome by gene targeting. Trends Genet 1989;5:70–6. [PubMed: 2660363]

Capecchi MR. Targeted Gene Replacement. Sci. Am 1994;270:52–59. [PubMed: 8134827]

Capecchi MR. Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. Nat .Rev 2005;6:507–512.

Cheah SS, Behringer RR. Contemporary gene targeting strategies for the novice. Molecular Biotechnology 2001;19:297–304. [PubMed: 11721625]

Cohen-Tannoudji M, Babinet C. Beyond ‘knock-out’ mice: new perspectives for the programmed modification of the mammalian genome. Mol. Hum. Reprod 1998;4:929–938. [PubMed: 9809673]

DeChiara TM. Gene targeting in ES cells. Methods Mol Biol 2001;158:19–45. [PubMed: 11236657]

Deng C, Capecchi MR. Reexamination of gene targeting frequency as a function of the extent of homology between the targeting vector and the target locus. Mol. Cell. Biol 1992;12:3365–3371. [PubMed: 1321331]

Doetschman T, Gregg RG, Maeda N, Hooper ML, Melton DW, Thompson S, Smithies O. Nature 1987;330:576–578. [PubMed: 3683574]

Dymecki SM. Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. Proc. Natl. Acad. Sci 1996;93:6191–6196. [PubMed: 8650242]

Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature 1981;292:154–156. [PubMed: 7242681]

Feil R, Wagner J, Metzger D, Chambon P. Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. Biochem Biophys Res Commun 1997;237:752–757. [PubMed: 9299439]

Ferradini L, Gu H, De Smet A, Rajewsky K, Reynaud CA, Weill JC. Rearrangement-enhancing element upstream of the mouse immunoglobulin kappa chain J cluster. Science 1996;271:1416–1420. [PubMed: 8596914]

Grimm D. A mouse for every gene. Science 2006;312:1862–1866. [PubMed: 16809501]

Gu H, Marth JD, Orban PC, Mossman H, Rajewsky K. Deletion of a DNA polymerase β gene segment in T cells using cell type-specific gene targeting. Science 1994;265:103–106. [PubMed: 8016642]

Hanks M, Wurst W, Anson-Cartwright L, Auerbach AB, Joyner AL. Rescue of the En-1 mutant phenotype by replacement of En-1 with En-2. Science 1995;269:679–682. [PubMed: 7624797]

Hasty P, Rivera-Pérez J, Bradley A. The length of homology required for ene targeting in embryonic stem cells. Mol. Cell. Biol 1991;11:5586–5591. [PubMed: 1656234]

Hasty P, Rivera-Pérez J, Bradley A. The role and fate of DNA ends for homologous recombination in embryonic stem cell. Mol. Cell. Biol 1992;12:2464–2474. [PubMed: 1588950]

Hasty, P.; Abuin, A.; Bradley, A. Gene Targeting: a Practical Approach. Joyner, AL., editor. Oxford University Press; New York: 1993. p. 1-35.

Hayashi S, McMahon AP. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. Dev. Biol 2002;244:305–318. [PubMed: 11944939]

Iredale JP. Gene knockouts. Mol. Pathol 1999;52:111–116. [PubMed: 10621830]

Jasin M, Moynahan ME, Richardson C. Targeted transgenesis. Proc. Natl. Acad. Sci 1996;93:8804–8808. [PubMed: 8799106]

Ledermann B. Embryonic stem cells and gene targeting. Exp. Physiol 2000;85.6:603–613. [PubMed: 11187956]

LeMouellic H, Lallemand Y, Brûlet P. Targeted replacement of the homeobox gene Hox-3.1 by the Escherichia coli lacZ in mouse chimeric embryos. Proc. Natl. Acad. Sci 1990;87:4712–4716. [PubMed: 1972279]

Lin FL, Sperle K, Sternber N. Recombination in mouse L cells between DNA introduced into cells and homologous chromosomal sequences. Proc. Natl. Acad. Sci 1984;82:1391–1395. [PubMed: 3856266]

Lu ZH, Brooks JT, Kaufman RM, Ley TJ. Long targeting arms do no increase the efficiency of homologous recombination in the β-globin locus of murine embryonic stem cells. Blood 2003;102:1531–1533. [PubMed: 12730107]

Mak, TW., editor. The Gene Knockout Factsbook. Academic Press; New York: 1998.

Majzoub JA, Muglia LJ. Knockout Mice. N Engl J Med 1996;334:904–7. [PubMed: 8596575]

Mansour SL, Thomas KR, Capecchi MR. Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature 1988;336:348–352. [PubMed: 3194019]

Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci 1981;78:7634–7638. [PubMed: 6950406]

McBurney MW, Sutherland LC, Adra CN, Leclair B, Rudnicki MA, Jardine K. The mouse Pgk-1 gene promoter contains an upstream activator sequence. Nucleic Acids Res 1991;19:5755–61. [PubMed: 1945853]

Melton DW. Gene-targeting strategies. Methods Mol Biol 2002;180:151–73. [PubMed: 11873649]

Mombaerts P, Clarke AR, Hooper ML, Tonegawa S. Creation of a large genomic deletion at the T-cell antigen receptor β-subunit locus in mouse embryonic stem cells by gene targeting. Proc. Natl. Acad. Sci 1991;88:3084–3087. [PubMed: 1826563]

Müller U. Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis. Mech. Dev 1999;82:3–21. [PubMed: 10354467]

Mouse Genome Sequencing Consortium. Initial sequencing and comparative analysis of the mouse genome. Nature 2002;420:520–562. [PubMed: 12466850]

Olson EN, Arnold HH, Rigby PW, Wold BJ. Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. Cell 1996;85:1–4. [PubMed: 8620528]

Rickert RC, Roes J, Rajewsky K. B lymphocyte-specific, Cre-mediated mutagenesis in mice. Nucleic Acid research 1997;25:1317–1318.

Rijkers T, Van Den Ouweland J, Morolli B, Rolink AG, Baarends WM, Van Sloun PPH, Lohman PHM, Pastink A. Targeted inactivation of mouse RAD52 reduces homologous recombination but not resistance to ionizing radiation. Mol. Cell. Biol 1998;18:6423–6429. [PubMed: 9774658]

Sargent RG, Wilson JH. Recombination and gene targeting in mammalian cells. Curr. Res. Mol Ther 1998;1:584–592.

Sauer B, Henderson N. Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase. The New Biologoist 1990;2:441.

Sauer B. Manipulation of transgenes by site-specific recombination: use of Cre recombinase. Methods in Enxymology 1993;225:890.

Sauer B, Henderson N. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proc. Natl. Acad. Sci 1998;85:5166–5170. [PubMed: 2839833]

Schwartzberg PL, Goff SP, Robertson EJ. Germ-line transmission of a c-abl mutation produced by targeted gene disruption in ES cells. Science 1989;246:799–803. [PubMed: 2554496]

Sikorski R, Peters R. Transgenics on the internet. Nature Biotech 1997;15:289.

Smithies O, Gregg RG, Boggs SS, Koralewski MA, Kucherlapati RS. Insertion of DNA sequences into the human chromosomal β-globin locus by homologous recombination. Nature 1985;317:230–234. [PubMed: 2995814]

Stacey A, Schnieke A, McWhir J, Cooper J, Colman A, Melton DW. Use of double-replacement gene targeting to replace the murine α-lactalbumin gene with its human counterpart in embryonic stem cells and mice. Mol. Cell. Biol 1994;14:1009–1016. [PubMed: 8289781]

Sternberg N, Hamilton D. Bateriophage P1 site-specific recombination. J. Mol. Biol 1981;150:467–486. [PubMed: 6276557]

te Riele H, et al. Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. Proc. Natl. Acad. Sci 1992;89:5128–5132. [PubMed: 1594621]

Thomas KR, Folger KR, Capecchi MR. High frequency targeting of genes to specific sites in the mammalian genome. Cell 1986;44:419–428. [PubMed: 3002636]

Thomas KR, Capecchi MR. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell 1987;51:503–512. [PubMed: 2822260]

Torres, RM.; Kühn, R. Laboratory protocols for conditional gene targeting. Oxford University Press; New York: 1997.

Tybulewicz VLJ, Crawford CE, Jackson PK, Bronson RT, Mulligan RC. Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-able proto-oncogene. Cell 1991;65:1153–1163. [PubMed: 2065352]

Utomo ARH, Nikitin AY, Lee WH. Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. Nature Biotech 1999;17:1091–1096.

Vanlancius V, Smithies O. Testing an “in-out” targeting procedure for making subtle genomic modifications in mouse embryonic stem cells. Mol. Cell. Biol 1991;11:1402–1408. [PubMed: 1996101]

Vasquez KM, Marburger K, Intody Z, Wilson JH. Manipulating the mammalian genome by homologous recombination. Proc. Natl. Acad. Sci 2001;98:8403–8410. [PubMed: 11459982]

Vogel G. A knockout award in medicine. Science 2007;318:178–179. [PubMed: 17932258]

Wong EA, Capecchi MR. Homologous recombination between coinjected DNA sequences peaks in early to mid-S phase. Mol. Cell. Biol 1987;7:2294–2295. [PubMed: 3600663]

Yagi T, Ikawa Y, Yoshida K, Shigetani Y, Takeda N, Mabuchi I, Yamamoto T, Aizawa S. Homologous recombination at c-fyn locus of mouse embryonic stem cells with use of diptheria toxin A-fragment gene in negative selection. Proc. Natl. Acad. Sci 1990;87:9918–9992. [PubMed: 2263643]

Zambrowicz BP, Sands AT. Knockouts model the 100 best-selling drugs — will they model the next 100? Nature Rev 2003;2:38–51.

Kmiec, EB., editor. Gene Targeting Protocols. Humanna Press; New Jersey: 2000. www.cellmigration.org/resource/komouse/komouse_resources_targeting4beginners.shtml

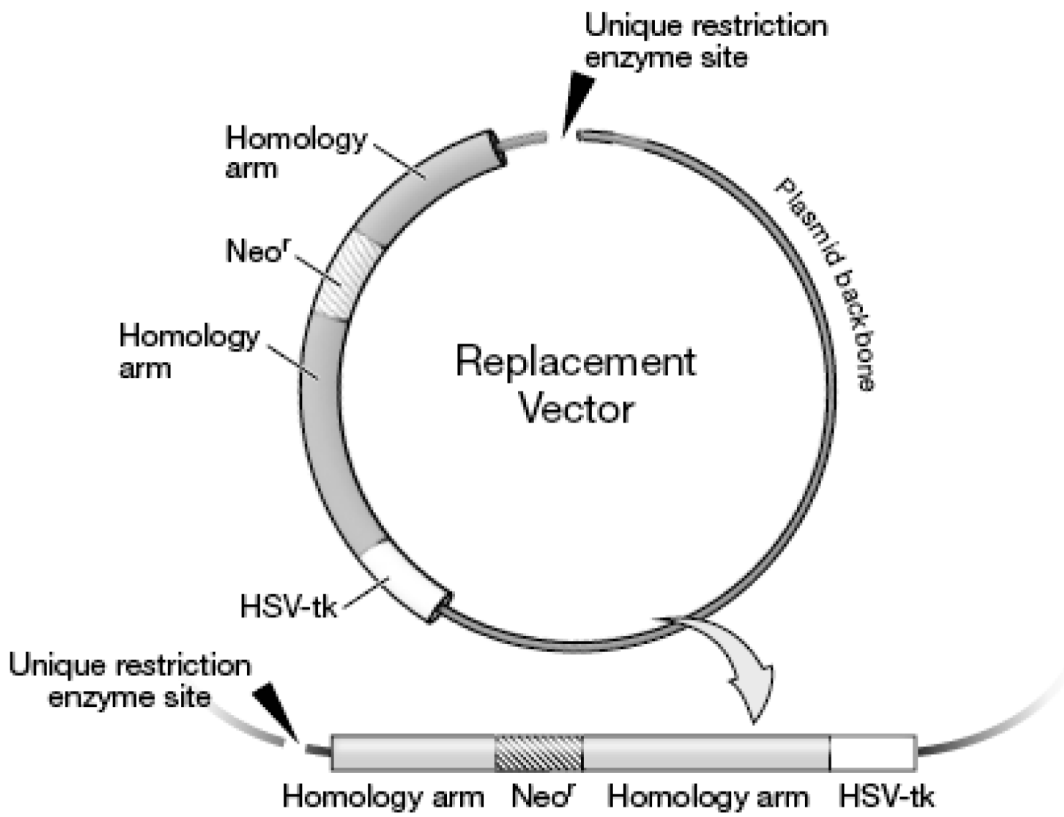

Figure 1.

A schematic of a replacement vector: Two homology arms flank a positive drug selection marker (neo^r). A negative selection marker (HSV-tk) is placed adjacent to one of the targeting arms. A unique restriction enzyme site is located between the vector backbone and the homology arm. When linearized for gene targeting, the vector backbone will then protect the HSV-tk from nucleases.

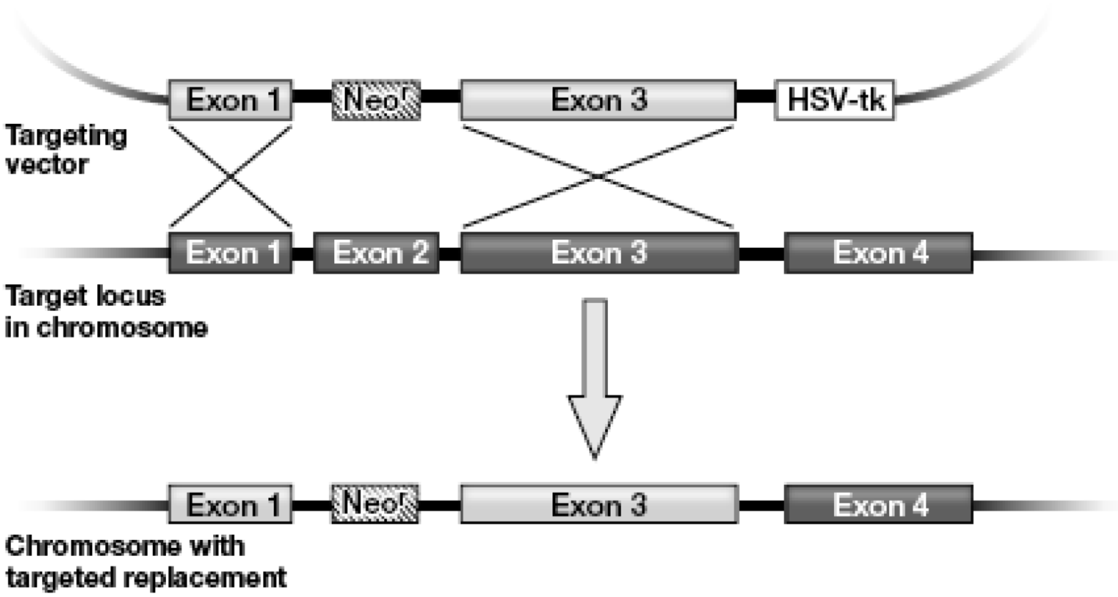

Figure 2.

Gene inactivation through a replacement vector: Homologous recombination with a replacement vector requires a positive selection marker (neo^r), a negative selection marker (HSV-tk), and two targeting arms (homologous sequence is depicted with exons in light grey). A representative target gene (with exons in dark grey) is aligned with the targeting vector. For this example, the targeting vector is designed so that Exon 2 is substituted with the neo^r gene. The replacement of Exon 2 by the neo^r gene is then recapitulated in the target locus as homologous recombination exchanges genomic sequence for the homologous sequence of the targeting vector. Two homologous recombination events (depicted through the crosses) occur via the long and short targeting arms to introduce non-homologous sequence (i.e., neo^r) into the designated gene. Insertion of the neo^r gene is selected for by treatment of cells with neomycin sulfate (G418) in tissue culture. The negative selection marker (HSV-tk) is not recombined into the chromosome and is lost during gene targeting. If the targeting construct is randomly integrated anywhere in the genome, the HSV-tk gene would be intact. Random integrants can be selected against through either gancyclovir or FIAU treatment.

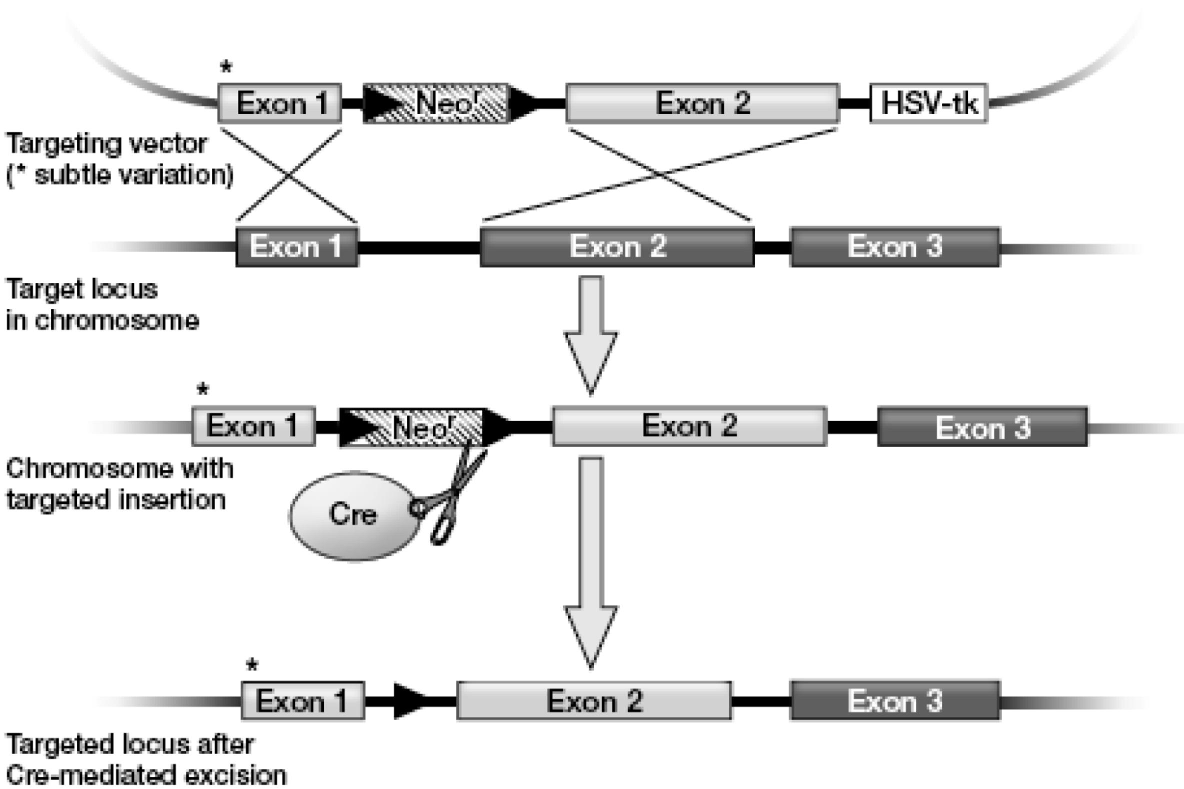

Figure 3.

Gene targeting to insert a subtle mutation: In the targeting vector, a subtle mutation (depicted as '*') is designed into one of the homology arms. Subtle mutations can consist of point mutations, micro-deletions, or insertions. When sequence in the target gene is exchanged with homologous sequence from the targeting vector, the subtle mutation is introduced into the chromosome. Homologous recombination occurs through both the long and short homology arms. A positive drug selection marker (neo^r) is needed in this strategy to select for clones that have undergone recombination. With a clean mutation, the neo^r gene is inserted into the targeted locus, but no exons are lost as a result of recombination. The neo^r gene needs to be removed so that it does not interfere with transcription of the recombined allele. LoxP sites (depicted as black triangles) are used to flank the neo^r gene in order to facilitate its removal. These 34 bp loxP sites are recognized by the Cre recombinase, which can be introduced into targeted stem cells by transient transfection. If the loxP sites are in the same direction, the Cre recombinase will circularize out any intervening sequence. With Cre-mediated recombination, only a single loxP site will remain. The loxP site in the recombined allele is situated within intron sequence so that it does not interfere with transcription of the mutant protein.

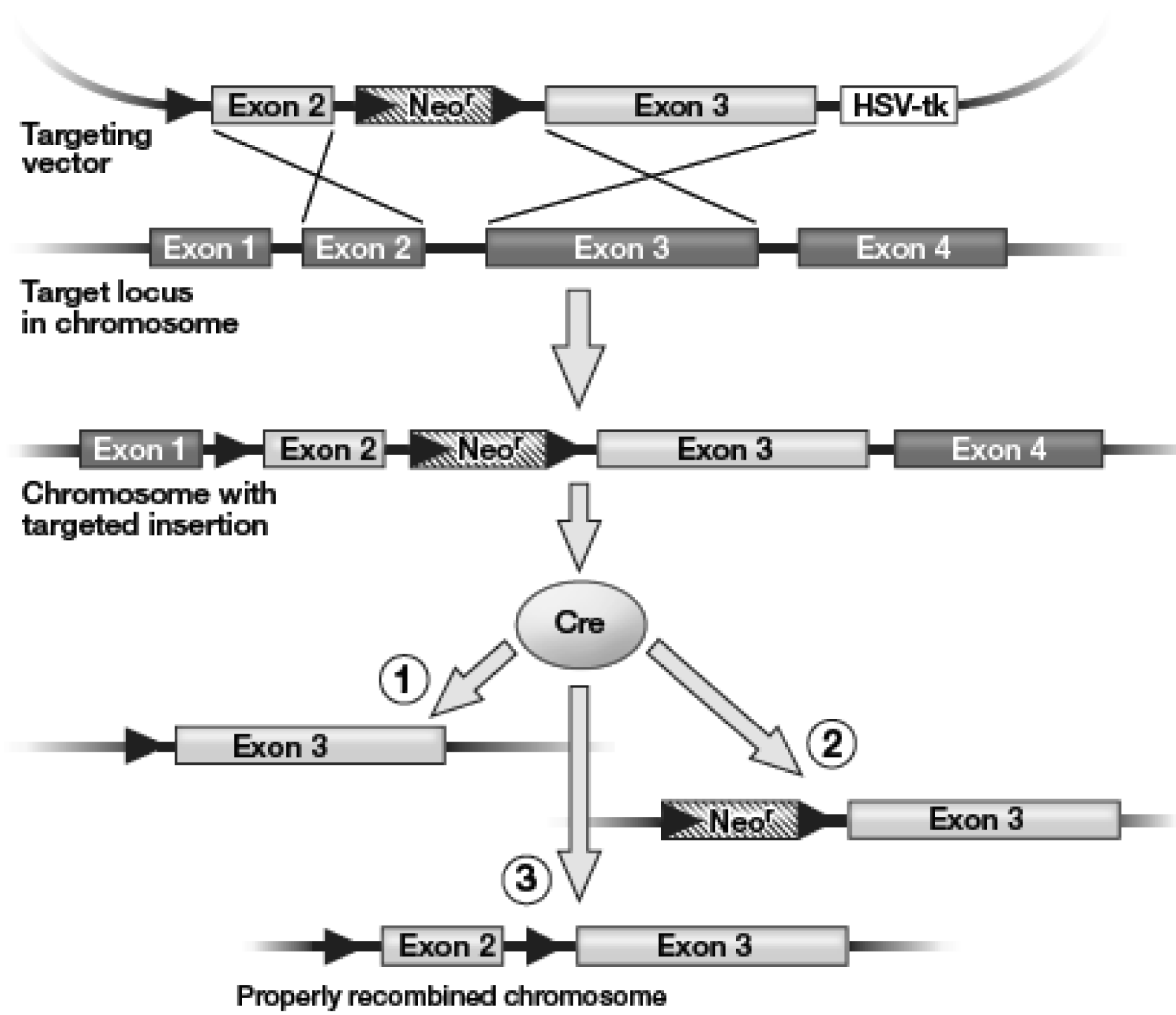

Figure 4.

Gene targeting to create a conditional knockout: In this strategy, the targeting construct is designed so that a loxP site (indicated with a black triangle) is located next to an essential exon within the homology arm. A floxed neo^r gene is then positioned on the opposite side of this exon. Both the loxP site and the floxed neo^r gene are introduced into the target locus through homologous recombination. Unlike a conventional knockout experiment, the targeting vector is assembled so that no exons are lost as a result of homologous recombination. After the stem cell clone with the properly targeted insertion is identified, the Cre recombinase is then introduced into the cells through a transient transfection. The Cre recombinase can produce three types of recombinations. In the first example (shown as ①), both Exon 2 and the neo^r gene are excised from the chromosome leaving only 1 loxP site. In the second scenario (shown as ②), only Exon 2 is removed while the floxed neo^r gene remains in the targeted chromosome. In the third case (shown as ③), just the neo^r gene is excised while 2 loxP sites remain to flank Exon 2, creating a floxed allele. Only the stem cell clones with this specific recombination are injected into blastocysts to generate floxed mice. Conditional deletion of Exon 2 in vivo is then accomplished typically by breeding the floxed mice with a Cre-expressing transgenic line. After the removal of neo^r in tissue culture and later the conditional deletion of Exon2 in vivo,

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the final allele has only one loxP site remaining within intron sequence of the targeted gene (as depicted in ①).

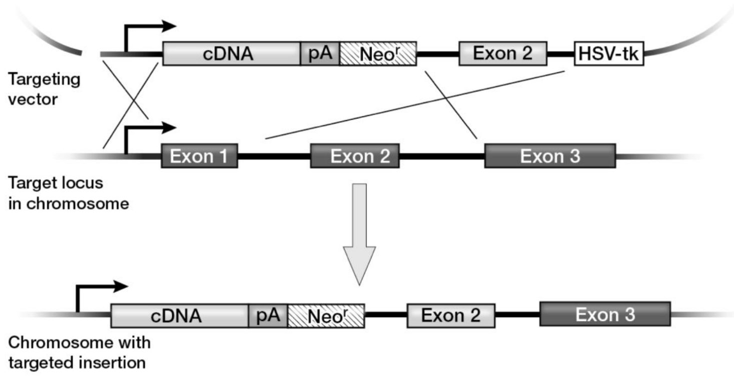

Figure 5.

Knock-in of a cDNA through the use of gene targeting: Knock-in constructs are similar in design to conventional knockout gene targeting vectors, except that additional sequence (i.e., a cDNA or protein domain of interest) is inserted into the target gene. Through homologous recombination, this foreign sequence is introduced in frame into the target locus to be expressed by its promoter. Essential coding sequence in the target locus is simultaneously lost during recombination with the targeting construct. In this example, homologous recombination places a cDNA under the control of the promoter of the target gene. Concurrently, the translational start sequence in Exon 1 of the target gene is also replaced by the cDNA. The cDNA, in this example, has a poly A addition signal (pA) which will stop any further transcription downstream of the targeted insertion. For a knock-in targeting vector, one of the homology arms must consist of genomic sequence upstream of the planned insertion site for the cDNA. To knock in a cDNA, as shown, a targeting vector must use promoter sequence for one of its homology arms (as depicted with the directional arrow). A positive drug selection marker (neo^r) is still needed to select for clones that have inserted the designated cDNA into the target gene. Two homologous recombination events serve to insert the cDNA and neo^r gene into the target location while knocking out Exon 1.

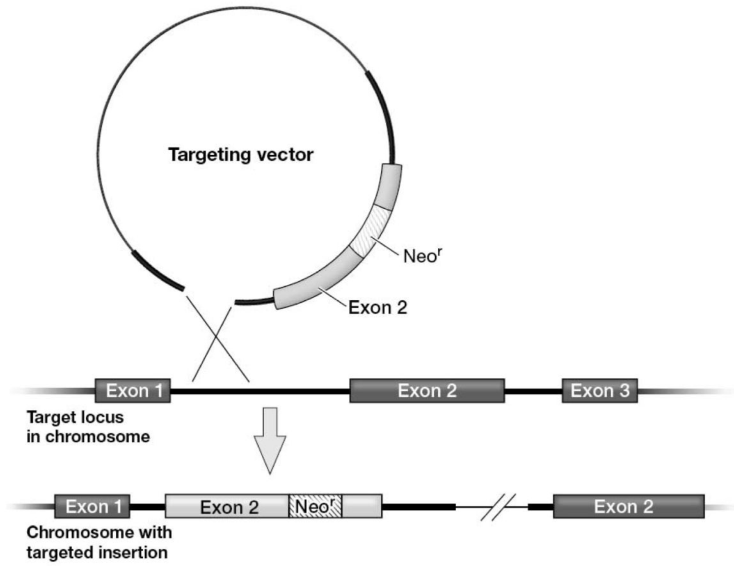

Figure 6.

Gene inactivation with an insertion vector: For an insertion vector, only one homologous recombination event is needed for targeted insertion of DNA into a designated gene. In this case, recombination occurs around a double strand break that is located in the homology arm of the targeting construct. The entire insertion vector is then incorporated into the gene, including the plasmid backbone (represented by the thin line). A drug selection marker like the neo^r gene is still needed for positive selection, but this marker can be positioned either in the targeting arm or in the plasmid backbone of the insertion vector. In this example, the positive drug selection marker is designed in the homology arm in order to replace essential coding sequence of the target gene (as shown with the disruption of Exon 2 by the neo^r gene). For insertion vectors, a knockout allele is essentially generated because the target gene is disrupted with insertion of the neo^r gene and by duplication of exonic sequence.